MALARIA MOSQUITO LARVAE IN COMPETITION FOR LIMITED RESOURCES

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Malaria Mosquito Larvae in Competition for Limited Resources

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The mosquitoes of the *Anopheles gambiae* species complex are the primary vectors of malaria in Africa. These *Anopheles* species demonstrate large geographical overlap and their larvae often inhabit the same small aquatic pools in nature. To understand how larval interactions between these mosquito species might affect development and success in the field, I studied larval competition between three members of the *An. gambiae* complex: *An. arabiensis, An. quadriannulatus,* and two strains of *An. coluzzii* (Suakoko and Mopti) in resource limited conditions. To quantify competitiveness I measured larval survivorship and time to adult emergence. When raised on a limited food diet, *An. arabiensis* demonstrated the lowest larval survivorship. Survivorship of *An. quadriannulatus, An. coluzzii* Mopti and *An. coluzzii* Suakoko was all significantly higher than *An. arabiensis,* but there was no significant difference among these three species. Surprisingly, when I competed *An. arabiensis* against *An. coluzzii* Suakoko and Mopti in these same conditions, it outcompeted both with significantly higher survivorship. Additionally, *An. quadriannulatus* and *An. arabiensis* survivorship increased when the two competed against each other compared to competition against siblings. The increased survivorship of *An. arabiensis* when in competition with *An. coluzzii* species suggests a competitive interaction is present that may influence their population sizes and ranges in the field.

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CHAPTER I

INTRODUCTION

Human malaria, mediated by the *Plasmodium* parasite, is transmitted by approximately 60 mosquito species of the *Anopheles* genus (Neafsey, et al., 2015). The *Anopheles* genus is responsible for the approximately 200 million new malaria cases each year with 90% of these cases occur across sub-Saharan Africa. Specific members of the *Anopheles gambiae* species complex are responsible for the majority of this malaria transmission in Africa. (World Malaria Report, 2016). Eight species make up the *Anopheles gambiae* species complex, and though morphologically identical, they show distinct variation in behavior that affects their vectorial capacity (Lehmann, et al., 2006). This variation warrants thorough investigation into the behaviors and characteristics of these *Anopheles* mosquitoes that make them such competent vectors of human malaria.

This project focuses on three members of the *An. gambiae* species complex: *An. arabiensis, An. coluzzii,* and *An. quadriannulatus. An. coluzzii* is a primary vector of malaria as it feeds exclusively on humans. *An. arabiensis* is considered another dominant vector because they opportunistically feed on humans and animals. *An. quadriannulatus* rarely feeds on humans, making it a minor malaria vector. This level of host-preference/specialization is fairly unique among mosquitoes. Additionally, these species show significant geographical overlap and similarities in habitat preference that likely lead to interactions among species (Coetzee, et al. 2000). However, there has been very little research into these interactions and how they may affect each species' development and success.

One stage in the *Anopheles* life cycle where different *Anopheles* species often interact is the immature stage. This portion of the life cycle consists of a progression through three aquatic

stages: egg, larva, and pupa. Eggs are laid in transient pools such as discarded containers, tire tracks or rice fields that have collected rain water. The availability of larval habitats and quantity of larvae directly correlates with rainfall (Fillinger, et al., 2004). After hatching, larvae develop through four larval instars over the course of 9-12 days before pupating. The mosquito remains at the pupal stage for 1-3 days before emerging into an adult and leaving the aquatic environment. The larval stage is critical to development as the nutrition necessary to transition to the pupal and adult stages is acquired during this stage.

Several factors can affect larval development and survivorship which include nutrient and larval density, intra- and inter-specific competition, temperature, and predation (Gimonneau et al., 2014). Of these factors, nutrient density is one of the most significant (Shapiro, et al. 2016). Previous work has shown variation in response to environmental conditions among the *Anopheles* species complex (Schneider et al., 2000). For example, *An. arabiensis* larvae demonstrate greater tolerance for high temperatures than *An. gambiae* (Kirby, et al., 2009). Since *Anopheles* breeding pools are temporary rather than permanent habitats like ponds and streams, the amount of nutrition available to the larvae can be very low. It is therefore possible that there is strong competition for limited food, both within and between species. For this project, I measure the effects of nutrient deprivation on larval development between three species of the Anopheles gambiae complex when raised in single or mixed species environments.

CHAPTER II

METHODS

Mosquitoes

The *Anopheles quadriannulatus* (strain: Sansqua), *Anopheles arabiensis* (strain: Dongola), and *Anopheles coluzzii* (strains: Mopti and Suakoko) mosquitoes were obtained through BEI Resources. All strains were reared in a climate controlled room at 85% relative humidity and 25°C with 12 light :12 dark hr photoperiod. Larvae were kept in plastic bins filled with distilled water and were fed ground fish food (TetraPro Tropical Crisps®). Pupae were removed from larval containers daily and placed in small plastic cups inside adult cages for emergence. Adults were given constant access to a 5% glucose diet and were blood-fed twice per week using defibrinated sheep blood from HemoStat Laboratories. Wet filter paper was placed inside the bin following the blood-feed and eggs were removed and placed in a plastic larval bin filled with distilled water.

Larval Rearing

One-day old larvae were removed from larval bins using a plastic pipette. Larvae were separated into water drops in a petri dish and counted using a standard dissecting microscope. A total of 50 larvae were placed in a 100x15 mm polystyrene disposable petri dish (VWR® International) with 32 mL distilled water. The food stock solution (0.112 g fish food/mL) was prepared weekly or as needed. Nutrient density was designed to achieve a target survival of 65-75% (Gilles, et al., 2011). Larvae were given 100 µL (11.2 g fish food) of the food stock on alternate days for a twenty-day duration. Daily monitoring of the replicates included removing new pupae from each dish, placing the pupae in adult mosquito containers labeled with the date

of pupation and replicate, and tracking the rate of emergence to adulthood. Once each adult emerged, the mosquito was removed from the container and placed in a -20°C freezer for ten minutes. The mosquito was then de-winged and, if necessary, stored for later species identification.

Single species competition experiments were conducted using 50 larvae of each single species or hybrid (including both directions of hybridization) in limited resource setting. The same resource-limiting conditions were then applied to mixed species competition experiments, which included 25 larvae of one species and 25 larvae of a different species. This was performed in six replicates for each competition.

Species Identification

DNA Extraction

Adult mosquitoes from mixed species competition replicates were stored in Eppendorf tubes and kept in a -20°C freezer. DNA extraction was performed on the whole body of each adult mosquito collected from all competition replicates. The InstaGene Matrix DNA extraction protocol was adapted from BIO-RAD as followed. The mosquito tissue was crushed in 1 mL of autoclaved nanopure water and allowed to incubate at room temperature for 30 minutes. It was then centrifuged at 12,000 rpm for 3 minutes, after which all but 20-30 µL of supernatant was removed. 200 µL of 6% InstaGene matrix, which had been mixing on a magnetic stirrer, was added and the mixture was incubated at 56℃ for 30 minutes. After removal from the incubator each mixture was vortexed for 10 seconds and placed in 90℃ water for 10 minutes. Lastly, each mixture was vortexed for an additional 10 seconds and centrifuged at 12,000 rpm for three minutes. The supernatant, which contained the purified DNA, was removed and stored at -20℃.

PCR-Species Diagnostic

A ribosomal DNA-polymerase chain reaction (PCR) method was adapted from the protocol established by Scott et al. (1993) and Fanello et al. (2002) for species identification of the *An. gambiae* species complex. The method is based on species-specific nucleotide sequences in the intergenic spacers (rDNA IGS) and the 28S coding region, and can be applied to extracted DNA or fragments of the mosquito. The rDNA PCR assay includes four primers: universal primer, *An. arabiensis* specific primer (315 bp PCR product), *An. coluzzii* specific primer (390 bp) and *An. quadriannulatus* specific primer (153 bp PCR product). PCR products were run on a 2% agarose gel containing 0.1% Gel-Red. DNA bands were visualized with UV light.

Wing Measurement

Both wings were removed on the day of adult emergence and affixed to a glass slide with transparent tape. The wings were measured using a standard dissecting microscope. A Moticam (CMOS) X microscope camera was used to take pictures of each set of wings. Measurements of each wing were recorded in millimeters using Motic Images Plus 2.0 software. Only one wing length measurement was recorded for mosquitoes for which only one undamaged wing was available, and if two undamaged wings were available for measurement the average of the two wing lengths was used in statistical analysis.

Statistical Analysis

Larval survival was analyzed using generalized linear mixed models on the proportion of larvae that successfully emerged. For the models, the response variable was the ratio of pupal or

adult success/failures, the fixed variable was species identity, and the random variable was replicate.

Adult mosquito size was analyzed by measuring wing length. A two-way analysis of variance (ANOVA) was applied to the control replicates to determine if there was a statistically significant difference in mean wing length among the species. This was followed by a post-hoc Tukey analysis (CI=0.95) to examine all possible pairwise comparisons. After the results of species identification were available, two-way ANOVA was conducted on the competition groups to observe if any significant difference in wing length between the species was present when they were placed in competition.

CHAPTER III

RESULTS

Single Species Competition

Survivorship and Time to Adult Emergence

Single species competition trials were completed for *An. arabiensis, An. coluzzii* Mopti, *An. coluzzii* Suakoko, and *An. quadriannulatus.* (Figure 1A) *An. arabiensis* had the shortest adult emergence time frame (days 9-14) while *An. coluzzii* Suakoko (days 10-19) and *An. coluzzii* Mopti (days 10-16) had adult emergence time frames more similar to *An. quadriannulatus. An. quadriannulatus* adults had the most variation in emergence timing, ranging from day 9 through 19.

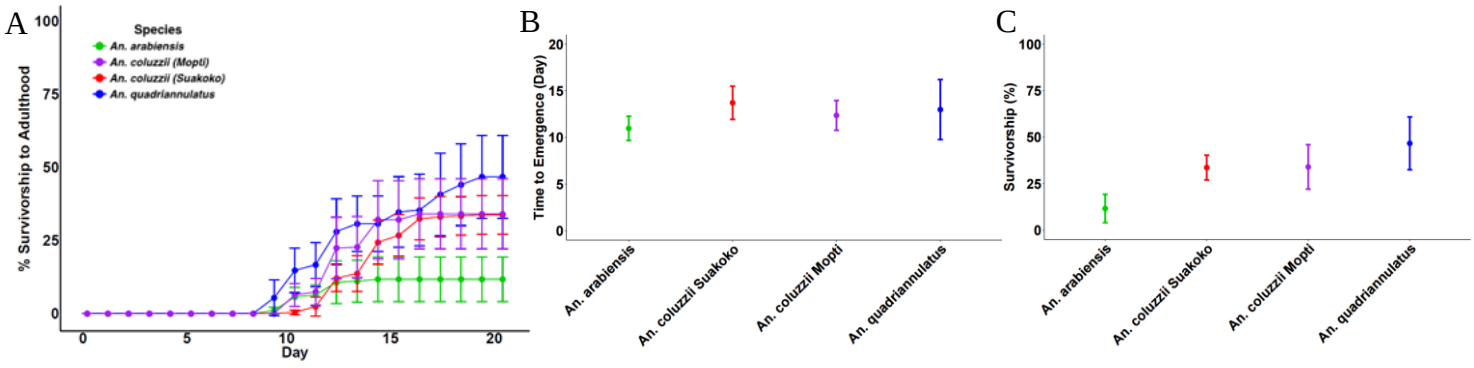

Figure 1: Survivorship to adulthood for *An. arabiensis, An. quadriannulatus, An. coluzzii* Mopti and *An. coluzzii* Suakoko. (A) The overall percent survivorship to adulthood for each species. (B) The time to emergence in days for each species. Data presented as mean and standard deviation.

There was also variation in the average time to emergence between *Anopheles* species raised on a reduced diet (Figure 1B). *An. quadriannulatus* and both strains of *An. coluzzii* emerged significantly later than *An. arabiensis.* Interestingly, they also had higher survivorship to adulthood, compared to *An. arabiensis* suggesting that time to emergence and development to adult may be linked in resource limiting conditions.

Applying the total emergence data on the final day (day 20) of each competition trial to a logistic regression model showed that at the adult stage (Figure 1C), *An. arabiensis* was significantly less successful reaching adulthood than *An. coluzzii* Suakoko, which had the next highest number of adults overall ($p<0.0001$). *An. quadriannulatus* and *An. coluzzii* Mopti had the greatest number of adults overall, but were not significantly greater than *An. coluzzii* Suakoko.

Mixed Species Competition

An. arabiensis and An. quadriannulatus

Raising *An. arabiensis* and *An. quadriannulatus* together in limited resources affected their development to adulthood compared to raising them separately (Figure 2A). *An. arabiensis* had lower success in the percentage of larvae that reached adulthood in both single ($p<0.001$) and mixed ($p=0.00147$) species competition compared to *An. quadriannulatus.*

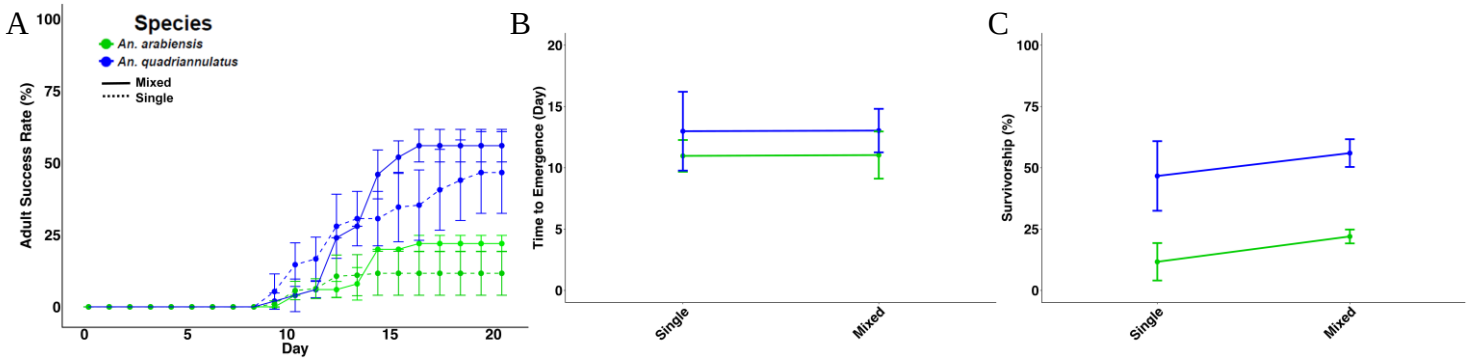

Figure 2: *An. arabiensis* and *An. quadriannulatus* in single and mixed species competition. (A) The average adult success rate of each species in twenty-day single and mixed competition trials. (B) Time to emergence and (C) percent survivorship for each species in single and mixed competition. Data presented as mean and standard deviation.

The two species were not impacted in larval time to adult emergence compared to single species competition trials (Figure 2B). Interestingly, both *An. quadriannulatus* ($p=0.0202$) and *An. arabiensis* ($p=0.0198$) improved in survivorship to adulthood compared to their success in

single species competition, suggesting that each species fares better in competition with the other species over their own (Figure 2C).

An. arabiensis and An. coluzzii Suakoko

Raising *An. arabiensis* and *An. coluzzii* Suakoko together in limited resources had a major impact on developmental success (Figure 3A). The two species were not impacted in larval time to adult emergence compared to single species competition trials (Figure 3B). In single species competition *An. arabiensis* had lower success in reaching adulthood than *An. coluzzii* Suakoko ($p<0.001$). However, in mixed species competition *An. arabiensis* were more successful than *An. coluzzii* Suakoko ($p<0.001$) (Figure 3C).

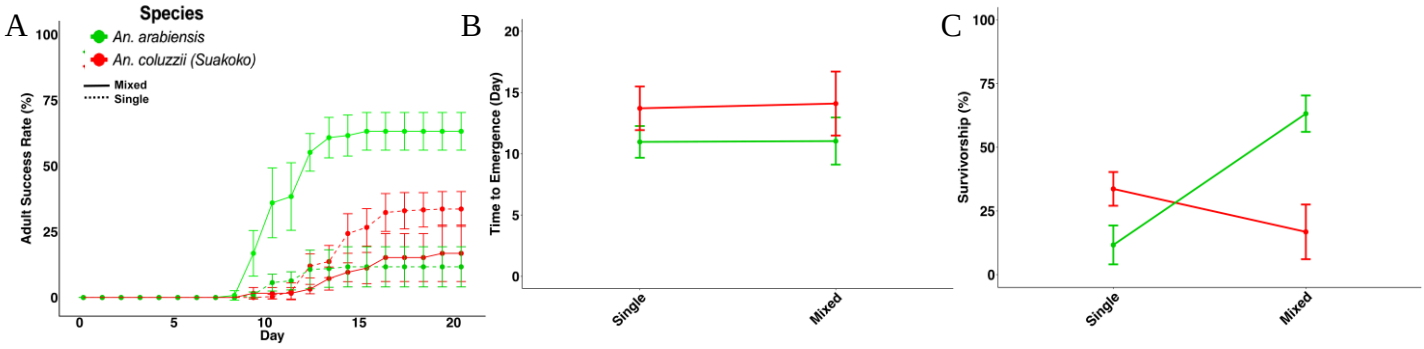

Figure 3: *An. arabiensis* and *An. coluzzii* Suakoko in single and mixed species competition. (A) The average adult success rate of each species in twenty-day single and mixed competition trials. (B) Time to emergence and (C) percent survivorship for each species in single and mixed competition. Data presented as mean and standard deviation.

The significant increase in survivorship of *An. arabiensis* in mixed species competition ($p<0.001$) suggests that a higher level of competition is present among *An. arabiensis* larvae, possibly due to advantageous differences in larval behavior in *An. arabiensis* that are not evident in other species. This competitiveness may be somewhat alleviated when in competition with *An. coluzzii* Suakoko. *An. coluzzii* Suakoko suffer in the presence of increased competitiveness by *An. arabiensis* compared to competing with other *An. coluzzii* Suakoko ($p=0.0425$). These

impacts on adult survivorship are opposite to what was observed in *An. arabiensis* and *An. quadriannulatus* mixed species competition.

An. arabiensis and An. coluzzii Mopti

An. arabiensis and *An. coluzzii* Mopti in mixed species competition for limited resources showed the same major impacts on survivorship that were seen in *An. arabiensis* / *An. coluzzii* Suakoko competition (Figure 4A). The two species were not impacted in larval time to adult emergence compared to single species competition trials (Figure 4B).

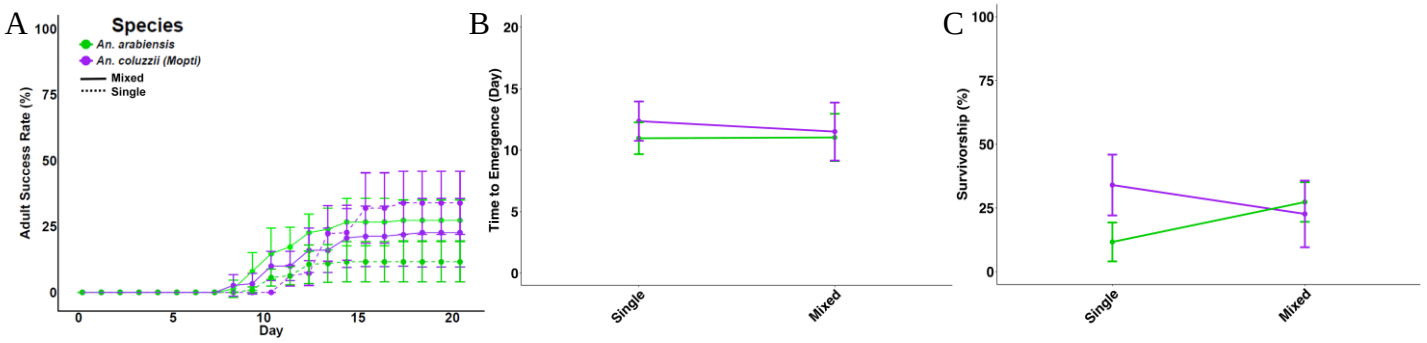

Figure 4: *An. arabiensis* and *An. coluzzii* Mopti in single and mixed species competition. (A) The average adult success rate of each species in twenty-day single and mixed competition trials. (B) Time to emergence and (C) percent survivorship for each species in single and mixed competition. Data presented as mean and standard deviation.

In single species competition, *An. arabiensis* had lower success in percentage of larvae that reached adulthood than *An. coluzzii* Mopti ($p<0.001$), but a reversal of success was again observed when the two were placed in competition. In mixed species competition *An. arabiensis* increased and *An. coluzzii* Mopti declined in survivorship, leading to statistically equivalent survivorships (Figure 4C). These impacts on adult survivorship are opposite what was observed in *An. arabiensis / An. quadriannulatus* competition but are the same trends observed in *An. arabiensis / An. coluzzii* Suakoko competition trials.

Species Wing Length Comparison

Wing length measurements were collected for all surviving adults from the *An. arabiensis, An. quadriannulatus,* and *An. coluzzii* (Mopti, Suakoko) single and mixed species competition trials. The wings were measured in millimeters and analyzed according to species and competition (Figure 5). All four species in single species competition were statistically equivalent in wing length. While the surviving mosquitoes of each species from mixed competition trials tended to have slightly longer wings than in single competition trials, this difference was not statistically significant. Male and female wing lengths were also compared for each species and no significant differences were found based on gender.

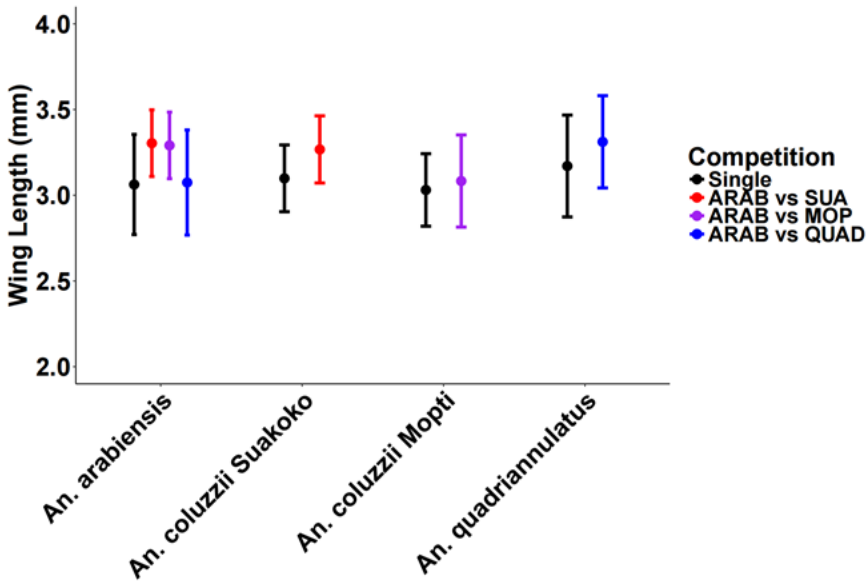

Figure 5: *An. arabiensis, An. coluzzii* Suakoko, *An. coluzzii* Mopti, and *An. quadriannulatus* wing length of surviving mosquitoes from single and mixed species competition. The average wing length of each species with standard deviation error bars.

CHAPTER IV

DISCUSSION

Comparison of adult survivorship between the four species in single and mixed competition environments identified unique interactions and trends among the species. In comparing success in single species competition, it appears that *Anopheles* species which take the longest time to emerge have the most success in resource limited conditions. *An. quadriannulatus* had the highest number of larvae reach the adult stage overall in single species competition and also showed the longest duration of adult emergence, while *An. arabiensis* had the shortest time frame of larvae emerging as adults and had the fewest number of larvae reach adulthood by the final day of the trial. Additionally, *An. arabiensis* mosquitoes had a relatively short range of time in which it emerged compared to the other *Anopheles* tested, suggesting it may have less plasticity in its developmental time and requirements. It is possible that greater competition exists among *An. arabiensis* larvae trying to emerge in this relatively short, rigid larval stage resulting in inadequate nutrient intake and lower overall survivorship to adulthood. This level of intraspecific competition did not result in such detrimental effects in the other three species.

Pairing the species in interspecific, mixed species competition trials identified differing interactions between species that resulted in varying levels of survivorship. Survivorship did not only differ between the two species in competition, but significant differences in survivorship were also present within each species between single and mixed species trials. Mixed species competition between *An. arabiensis* and *An. quadriannulatus* did not have a major effect on time to emergence of these two Anopheles species, however both species significantly improved when in mixed species competition as compared to when each species was competing only

amongst themselves in single species competition. These results suggest that *An. quadriannulatus* and *An. arabiensis* are of similar competitiveness in resource limiting conditions.

Surprisingly, when *An. arabiensis* were placed in competition with the *An. coluzzii* strains, *An. arabiensis* performance increased while *An. coluzzii* declined. *An. arabiensis* had the lowest overall survivorship to adulthood when competing against other *An. arabiensis* but had the greatest percent survivorship when competing directly with *An. coluzzii.* This reinforces that *An. arabiensis* experience a high level of competition when competing amongst themselves, but the decline of *An. coluzzii* was a different trend than was seen in *An. arabiensis / An. quadriannulatus* competition, where both species benefitted.

The enhanced success of *An. arabiensis* and simultaneous decline of *An. coluzzii* could be related to differences in larval behaviors that are advantageous in resourced limited environments, such as foraging ability or tolerance to delayed pupation. Large geographical overlap and more similar host-related behaviors between these two species might lead to them co-inhabiting the same aquatic habitats more frequently than either species would with *An. quadriannulatus,* causing this unique relationship between *An. arabiensis* and *An. coluzzii.* These two species may have adapted different responses to nutrient deprivation leading to unique interactions when placed in competition.

The wing length data for all four species in single and mixed species competition demonstrated no effect of competition on average wing length of any of the four species. It was originally hypothesized that if competition were to effect body size, shorter wing lengths would accompany lower survivorship. However, statistical analysis did reveal any significant effects of competition on wing length.

While these results show that there is variation in larval competition for limited resources among members of the *An. gambiae* complex, it is unclear how these interactions may influence mosquito populations in the field. However, our results warrant further investigation into the effects of interactions between *Anopheles* species on *Anopheles* ecology and malaria transmission in the field.

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